Dimerization of the cellular prion protein inhibits propagation of scrapie prions

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A central step in the pathogenesis of prion diseases is the conformational transition of the cellular prion protein (PrPC) into the scrapie isoform, denoted PrPSc. Studies in transgenic mice have indicated that this conversion requires a direct interaction between PrPC and PrPSc; however, insights into the underlying mechanisms are still missing. Interestingly, only a subfraction of PrPC is converted in scrapie-infected cells, suggesting that not all PrPC species are suitable substrates for the conversion. On the basis of the observation that PrPC can form homodimers under physiological conditions with the internal hydrophobic domain (HD) serving as a putative dimerization domain, we wondered whether PrP dimerization is involved in the formation of neurotoxic and/or infectious PrP conformers. Here, we analyzed the possible impact on dimerization of pathogenic mutations in the HD that induce a spontaneous neurodegenerative disease in transgenic mice. Similarly to wildtype (WT) PrPC, the neurotoxic variant PrP(AV3) formed homodimers as well as heterodimers with WTPrPC. Notably, forced PrP dimerization via an intermolecular disulfide bond did not interfere with its maturation and intracellular trafficking. Covalently linked PrP dimers were complex glycosylated, GPI-anchored, and sorted to the outer leaflet of the plasma membrane. However, forced PrPC dimerization completely blocked its conversion into PrPSc in chronically scrapie-infected mouse neuroblastoma cells. Moreover, PrPC dimers had a dominant-negative inhibition effect on the conversion of monomeric PrPSc. Our findings suggest that PrPSc monomers are the major substrates for PrPSc propagation and that it may be possible to halt prion formation by stabilizing PrPC dimers.

Prion diseases in humans and other mammals are characterized by a conformational transition of the cellular prion protein (PrPC)5 into an aberrantly folded isoform, designated scrapie prion protein (PrPSc). PrPSc can form amyloid plaques in the diseased brain and is the major constituent of infectious prions (for reviews, see Refs. 1–4). Propagation of PrPSc is strictly dependent on the synthesis of PrPC by the host (5) and involves a direct interaction of the two conformers (6, 7). Both mature PrPC and PrPSc contain a glycosylphosphatidylinositol (GPI) anchor and two N-linked carbohydrate moieties of complex structure, indicating that the conversion takes place after trafficking of PrPC through the secretory pathway at the plasma membrane or within endocytic compartments (8–12). However, both post-translational modifications are not required for PrPSc formation. Studies in prion-infected cultured cells and transgenic mice indicated that PrPC devoid of N-linked glycans still supports PrPSc propagation and formation of infectious prions (13). Similarly, transgenic mice expressing a secreted version of PrPC by deleting the C-terminal GPI anchor signal peptide (PrPΔGPI) propagate infectious prions after infection (14, 15).

Based on transgenic studies published by Prusiner and coworkers (6, 7), in 1991 John Hardy (16) proposed a model for the propagation of prions involving the formation of a PrPC/PrPSc heterodimer as an initial and essential step. Furthermore, he speculated that PrPC exists under physiological conditions...
as a dimer that has to dissociate before monomeric PrP<sub>C</sub> can interact with and is converted by PrP<sub>Sc</sub> (16). Indeed, we and others have provided experimental evidence for the existence of PrP<sub>C</sub> dimers in vitro and in vivo (17–20) with the internal hydrophobic domain (HD) as a putative dimerization domain (21). In this context, it might be interesting to note that in scrapie-infected cells only a small fraction of PrP<sub>C</sub> is converted into PrP<sub>Sc</sub>, indicating that not all PrP<sub>C</sub> molecules are suitable substrates for the conversion into PrP<sub>Sc</sub> (12).

To address the possibility that alterations in dimerization of PrP might be implicated in the formation of pathological PrP conformers, we investigated the activity of pathogenic PrP mutants to dimerize and analyzed the conversion of PrP<sub>C</sub> dimers into PrP<sub>Sc</sub>. Our study revealed that a pathogenic PrP mutant dimerizes similarly to WTPrP<sub>C</sub>; moreover, mutant PrP forms heterodimers with wildtype (WT) PrP<sub>C</sub>. Strikingly, stabilizing PrP<sub>C</sub> dimers prevented their conversion into PrP<sub>Sc</sub> in scrapie-infected neuroblastoma cells and inhibited endogenous prion propagation in trans.

**Results**

**A neurotoxic mutation does not interfere with homodimerization of PrP**

The formation of PrP<sub>C</sub> dimers was reported previously (17–19), and the internal HD has been identified as a putative dimerization domain (21). To address a possible role of PrP dimerization in the formation of a neurotoxic PrP conformer, we studied dimerization of PrP(AV3) in mouse neuroblastoma (N2a) cells. PrP(AV3) contains three alanine-to-valine changes within the HD (Fig. 1A) and causes early onset neurodegeneration upon expression in transgenic mice (22).

To force formation of PrP dimers, we replaced serine 132 by cysteine in PrP(AV3) (Fig. 1A). In case PrP dimerizes, an intermolecular disulfide bond can be formed that is stable in SDS buffer under nonreducing conditions (Fig. 1B). This approach was successfully used to study dimerization of the transmembrane receptor ErbB-2/Her2 (23), the amyloid precursor protein (24), and WTPrP<sub>C</sub> (19). Indeed, Western blot analysis of transiently transfected N2a cells revealed that similarly to WTPrP<sub>C</sub> the neurotoxic PrP(AV3) mutant forms homodimers that disassemble in the presence of reducing agents, such as β-mercaptoethanol (ME) (Fig. 1C). The ratio of monomeric/dimeric PrP species was stable at various expression levels, indicating that dimer formation was not an artifact of PrP overexpression (Fig. 1D).

PrP is characterized by a series of post-translational modifications. It is modified by two N-linked glycans of complex structure, a C-terminal GPI anchor, and an internal disulfide bond (for a review, see Ref. 25). To study whether forced dimerization interferes with maturation and/or cellular trafficking, we performed an indirect immunofluorescence analysis of cells transiently transfected with our PrP constructs. The staining pattern did not reveal obvious differences between the PrP variants containing a cysteine or serine at position 132, indicating that disulfide bond–linked dimers of WTPrP<sub>C</sub> and PrP(AV3) are transported through the secretory pathway (Fig. 2A, permeabilized) and are localized at the outer leaflet of the plasma membrane (Fig. 2A, non-permeabilized). To analyze the cell surface localization of PrP dimers in more detail, transfected cells were treated either with trypsin to remove extracellular domains of membrane-anchored proteins in general or with phosphatidylinositol-specific phospholipase C (PIPLC) to specifically liberate GPI-anchored proteins. As shown by Western blot analysis, trypsin treatment of live cells significantly reduced the signals of both PrP monomers and dimers in cell lysates, confirming that PrP dimers had been located at the plasma membrane (Fig. 2B). The cytosolic protein GAPDH was not affected by trypsin, verifying that the protease only digested extracellular proteins (Fig. 2B). Similarly, after incubation with PIPLC, monomeric as well as dimeric PrP was present in the cell culture media (Fig. 2C), demonstrating that the PrP dimers were inserted in the plasma membrane via a GPI anchor. Another post-translational modification of PrP is the conversion of the two N-linked glycans into complex structures (11). To evaluate the glycosylation status of PrP in detail, cell lysates were treated with endoglycosidase H (EndoH), which only cleaves high-mannose N-linked glycans, or peptide:N-glycosidase F (PNGaseF), which cleaves high-mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. Forced dimerization via the introduced cysteine residue did not interfere with complex glycosylation because the electrophoretic mobility of PrP was only increased after PNGaseF (Fig. 2D, left panel) but not after EndoH digestion (Fig. 2D, right panel). Notably, the enzyme reaction buffer contains reducing agent. Thus, only monomeric PrP is seen in Western blot analysis after EndoH or PNGaseF digestion. If PrP dimers were modified with high-mannose glycans only, an additional band would appear in the EndoH-treated samples.

This analysis revealed that neurotoxic mutations in the hydrophobic domain do not interfere with the dimerization of PrP. In addition, our data indicated that an engineered intermolecular disulfide bond between the hydrophobic domains of two PrP molecules does not impair maturation and cellular trafficking. Like WTPrP<sub>C</sub>, covalently linked PrP dimers are complex glycosylated and anchored to the outer leaflet of the plasma membrane via a GPI anchor.

**A neurotoxic PrP mutant forms heterodimers with WT PrP<sub>C</sub>**

To analyze whether the mutations in the HD might interfere with the formation of PrP(AV3)/WTPrP heterodimers, we inserted an HA epitope tag into WTPrP<sub>C</sub> and a V5 epitope tag into PrP(AV3). Both proteins contain a cysteine at position 132 to allow intermolecular disulfide bond formation (Fig. 3A). N2a cells transiently coexpressing WTPrP-HA and PrP(AV3)-V5 were lysed and subjected to immunoprecipitation with anti-HA antibodies under nonreducing conditions. The immunopellet was then analyzed by Western blotting using anti-V5 antibodies. PrP(AV3)-V5 copurified with WTPrP-HA, indicative of the formation of PrP heterodimers (Fig. 3B and C). As a control, anti-HA immunoprecipitations were performed in lysates from cells that express either HA-tagged WTPrP<sub>C</sub> or V5-tagged PrP(AV3). In neither case was V5-positive PrP detected in the immunopellets.

The formation of disulfide bond–stabilized PrP homo- and heterodimers indicated that PrP<sub>C</sub> has an intrinsic propensity to at least transiently undergo homotypic interactions in the
PrP dimerization inhibits prion formation

**A**

wtPrP

PrP(AV3)

A113V, A115V, A118V

**B**

+ ME

PrP-S-S-PrP

- ME

PrP-S-S-PrP

**C**

+ ME

wt PrP

AV3

% Dimerization

wt PrP

PrP(AV3)

ns

**D**

wtPrP

PrP(AV3)

% Dimerization

ns ns ns
secretory pathway of neuronal cells. To assess the dimerization under physiological conditions, we performed native immunoprecipitation assays with HA- and V5-tagged PrP constructs that did not include the Cys-132 mutation (Fig. 4A). Cells were cotransfected with an HA- together with a V5-tagged PrP construct, and cell lysates were subjected to immunoprecipitation under native conditions with anti-HA antibodies. The resulting immunopellet was then analyzed by Western blotting using anti-V5 antibodies. Using this approach, we could verify the formation of homodimers of WTPrPC and PrP(AV3) under physiological conditions (Fig. 4B, wt-HA + wt-V5 or AV3-HA + AV3-V5). Moreover, we could also detect heterodimers formed between WTPrPC and PrPSc(AV3) (Fig. 4B, wt-HA + AV3-V5). In lysates prepared from cells that express only V5- or HA-tagged PrP, no specific signals were detectable in Western blot analysis, demonstrating the specificity of the assay.

**Dimerization of PrPSc blocks formation of PrPSc**

Encouraged by the finding that maturation and intracellular trafficking of covalently linked PrP dimers are comparable with those of native PrPSc, we tested whether PrP dimers can be converted into PrPSc. To this end, we used scrapie-infected mouse neuroblastoma (ScN2a) cells that propagate PrPSc. Under reducing conditions (Laemmli sample buffer containing ME), only PrP monomers are detectable (Fig. 5, A and B) because ScN2a cells had already accumulated PrPSc prior to the expression of the PrPSc dimers, and PrPSc has a half-life time >24 h. As another control, we generate an alanine variant of PrPsc (PrP-A132). After transient expression in ScN2a cells, it was converted into PK-resistant PrPSc, indicating that the mutation of the serine residue does not prevent conversion into PrPSc (Fig. S1). In conclusion, the experiments in ScN2a cells revealed that dimeric PrPSc is not converted into PrPsc and has a dominant-negative effect on PrPSc propagation in trans.

**Discussion**

Dimerization of cell surface receptors is often associated with their physiological function. Our study emphasizes a propensity of the cellular prion protein to form dimers at the plasma membrane. Furthermore, we show that neurotoxic mutations within the hydrophobic domain do not interfere with the formation of homodimers or heterodimers between mutant PrP and WTPrPC. However, in contrast to monomeric PrP, covalently linked PrPdimers are not converted into PrPSc in scrapie-infected neuroblastoma (ScN2a) cells and inhibit prion propagation in trans.

The biological function of PrPSc still remains enigmatic, but various studies suggest a role of PrPSc as a cell surface receptor in stress-protective and neurotoxic signaling pathways (19, 31–36). Because receptors often form dimers, it is interesting to note that dimerization of PrPSc has been described in vitro and in vivo (17–20). Using disulfide bridge-mediated dimerization, we first corroborated these studies and showed that covalently linked dimers of PrP are complex glycosylated and GPI-an-
PrP dimerization inhibits prion formation

A

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wtPrP

PrP(AV3)

B

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Trypsin

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anti-GAPDH

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PIPLC

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PNGaseF

EndoH
chored to the outer leaflet of the plasma membrane. The introduction of an artificial disulfide bond allowed us to study a possible impact of dimerization on PrP maturation in the secretory pathway and to compare dimer formation between WT and mutant PrP. Specifically, these approaches revealed that at least 60% of total PrP dimerizes. Moreover, maturation and cellular trafficking of covalently linked PrP dimers were not altered compared with WTPrP<sub>C</sub>. Thus, neither the quality control machinery nor the glycan-modifying enzymes in the secretory pathway regarded PrP dimers as nonphysiological conformers. Finally, native immunoprecipitation assays provided evidence for the formation of WTPrP<sub>C</sub>-dimers under physiological conditions.

We observed that three mutations within the HD that induce the formation of neurotoxic PrP conformers (AV3) did not interfere with PrP dimerization, suggesting that the toxic potential of PrP<sub>AV3</sub> is not linked to alterations in dimer formation. However, we only analyzed dimer formation of the secreted form of PrP<sub>AV3</sub> and not of its transmembrane isoform, denoted PrP<sub>C</sub>-<sub>trans</sub> (22). Our finding that PrP<sub>AV3</sub> interacts and forms stable heterodimers with WTPrP<sub>C</sub> might be relevant for the observation that WTPrP<sub>C</sub> can prevent the toxic activity of PrP mutants lacking the HD (PrP<sub>D</sub>H<sub>D</sub>) (37). It is conceivable that WTPrP<sub>C</sub> via a direct interaction either blocks aberrant binding of PrP<sub>D</sub>H<sub>D</sub> to cellular proteins or prevents formation of a channel-forming PrP<sub>D</sub>H<sub>D</sub> conformer (38).

One of the most important findings of our study was the resistance of forced PrP<sub>C</sub> dimers to be converted into PrP<sub>Sc</sub> and their activity to interfere with endogenous PrP<sub>Sc</sub> propagation in trans. After transient expression in Scn2a cells, WTPrP<sub>C</sub> as well as PrP<sub>AV3</sub> was converted into PK-resistant PrP<sub>Sc</sub>. The dimeric variants of both proteins were expressed; however, they were not converted into PrP<sub>Sc</sub>. Moreover, the PrP<sub>C</sub> dimers inhibited the conversion of the monomeric fraction of the transfected PrP constructs and of endogenous mouse PrP<sub>C</sub> in trans. It was reported previously that a secreted artificial PrP-immunoglobulin Fc (PrP-Fc<sub>J</sub>) fusion protein that forms disulfide bond–stabilized dimers was not converted into PrP<sub>Sc</sub> and delayed onset of prion disease in transgenic mice (39). However, this study did not include the analysis of a variant of PrP-Fc<sub>C</sub> deficient in disulfide bond formation that would have been secreted as a monomeric PrP-Fc<sub>C</sub> fusion protein. Thus, it remains unclear whether the observed effects were due to the dimerization of PrP-Fc<sub>C</sub> or the expression of an artificial PrP-immunoglobulin fusion protein.

How can dimerization of GPI-anchored PrP<sub>C</sub> interfere with PrP<sub>Sc</sub> propagation (Fig. 5E)? Seminal studies in transgenic mice strongly suggested that PrP<sub>C</sub> directly interacts with PrP<sub>Sc</sub> to initiate propagation (6, 7). Based on these findings, John Hardy (16) proposed in 1991 that PrP<sub>C</sub> exists as a dimer in equilibrium with monomeric PrP<sub>C</sub>. He hypothesized that only PrP<sub>C</sub> monomers interact with PrP<sub>Sc</sub> and are converted by PrP<sub>Sc</sub>, whereas PrP<sub>C</sub> dimers are protected (16). Our study addressed this hypothesis experimentally. By stabilizing PrP<sub>C</sub> dimers via the intermolecular disulfide bond, dissociation is prevented, thereby decreasing the fraction of PrP<sub>C</sub>-monomers that can serve as PrP<sub>Sc</sub> substrates for conversion. However, the inhibitory effect of dimeric PrP<sub>C</sub> on the conversion of monomeric PrP<sub>C</sub> points to a more complex scenario. In particular, one can envision the following, mutually not exclusive pathways. (i) PrP<sub>C</sub> dimers do not bind to PrP<sub>Sc</sub> and therefore are not converted. This mode of action does not provide an obvious explanation for the inhibitory effect of dimeric PrP<sub>C</sub> on the conversion of monomeric PrP<sub>C</sub>. (ii) Dimeric PrP<sub>C</sub> still interacts with PrP<sub>Sc</sub>; however, such interactions do not lead to the de novo formation of PrP<sub>Sc</sub> either because the energy barrier for the conversion of PrP<sub>C</sub> dimers is too high or because the interaction surface between the PrP<sub>C</sub>-dimer and PrP<sub>Sc</sub> is different and not suited to initiate or complete conversion. Because we artificially introduced a covalent linkage to stabilize PrP<sub>C</sub> dimers, one cannot rule out that this modification and not dimerization of PrP<sub>C</sub> blocked the conversion. In particular, the artificial cross-link could limit the structural mobility of the protein in ways that a native dimer may not, resulting in the inhibition of prion conversion. (iii) PrP<sub>C</sub> dimers bind to PrP<sub>Sc</sub> with a higher affinity than monomeric PrP<sub>C</sub>. As a consequence, interaction of monomeric PrP<sub>C</sub> with PrP<sub>Sc</sub> is decreased, and its conversion is reduced. (iv) PrP<sub>Sc</sub> in complex with PrP<sub>C</sub> dimers is subjected to increased intracellular degradation. It will now be interesting to explore the therapeutic potential of substances that can stabilize native PrP<sub>C</sub> dimers and hence block the conversion process.

**Experimental procedures**

**Plasmids**

Plasmid amplification and maintenance were carried out in *Escherichia coli* TOP10<sup>®</sup> (Thermo Fisher Scientific). The murine prion protein (GenBank<sup>TM</sup> accession number M18070) was modified to express PrP<sub>L108M/V111M</sub> (40), allowing detection by the mAb 3F4 (29). The amino acid numbers refer to the human prion protein. All constructs generated for this study were developed by standard PCR cloning techniques. In some constructs, serine 132 was mutated to alanine or cysteine to enable the formation of a disulfide-bonded dimer (denoted A132 or C132). PrP<sub>AV3</sub> (A113V/A115V/A118V) was cloned...
PrP dimerization inhibits prion formation

A

wtPrP-HA

PrP(AV3)-V5

AGAAAAGAVGGLGYYMLGC

VGAAAAGAVGGLGYYMLGC

B

1. IP: anti-HA

2. WB: anti-V5

C

IP: anti-HA

WB: anti-V5

Input anti-HA

Input anti-V5

kDa

50

35

*dimer

*w

<monomer

50

35

*dimer

<monomer

*
from a plasmid encoding PrP(AV3,L9R) kindly provided by David Harris (41). All constructs described above were inserted into pcDNA3.1/Neo (+) vector (Invitrogen). If indicated, PrP mutants were equipped with a V5 tag (GGTAAACCGATACCG-AACCCGCTCCTCGGTCTCGATTCGACG) or HA tag (TACC-CATACGATGTTCCAGATTACGCT) inserted in the unstructured N-terminal region between amino acids 35 and 36.

Antibodies and reagents

The following antibodies were used: anti-PrP monoclonal antibodies 3F4 (29) and 4H11 (42), mouse monoclonal anti-V5 antibody (mAb R960CUS, Thermo Fisher Scientific), anti-HA (mAb MMS-101R, Covance), mouse monoclonal anti-GAPDH (mAb AM4300, Thermo Fisher Scientific), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Thermo Fisher Scientific), and IRDye-conjugated secondary antibody (IRDye 800CW donkey anti-mouse, LI-COR Biosciences). All standard chemicals and reagents were purchased from Sigma-Aldrich if not otherwise noted. The following reagents were used: EndoH (New England Biolabs), PNGaseF (New England Biolabs), PIPLC (Thermo Fisher Scientific), trypsin (Thermo Fisher Scientific), monoclonal anti-HA-agarose beads (Sigma-Aldrich), cOmplete™ Mini EDTA-free Protease Inhibitor Mixture (Roche Applied Science).

Cell lines, transfection, and lysis

Human HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX (Thermo Fisher Scientific).

Figure 3. The neurotoxic mutant PrP(AV3) forms disulfide bond–linked heterodimers with WTPrP. A, schematic representation of the constructs used. The cysteine variants of WTPrP and PrP(AV3) were modified with an HA (WTPrP-HA and PrP(AV3)-HA) and a V5 tag (WTPrP-V5 and PrP(AV3)-V5). The tags were inserted after amino acid 35. B, HeLa cells were transiently transfected with the indicated constructs. Cells were lysed, and HA-tagged PrP was immunoprecipitated under nonreducing conditions with HA-agarose beads. The immunopellet was dissolved in Laemmli sample buffer containing β-mercaptoethanol and analyzed by Western blotting (WB) using an anti-V5 antibody. Western blot analysis of the inputs is shown below. Asterisk, signal corresponds to primary antibody used in the immunoprecipitation (IP).

Figure 4. The neurotoxic mutant PrP(AV3) forms heterodimers with WTPrP under physiological conditions. A, schematic representation of the constructs used. The serine variants of WTPrP and PrP(AV3) were modified with an HA (WTPrP-HA and PrP(AV3)-HA) and a V5 tag (WTPrP-V5 and PrP(AV3)-V5). The tags were inserted after amino acid 35. B, HeLa cells were transiently transfected with the indicated constructs. Cells were lysed, and HA-tagged PrP was immunoprecipitated under nonreducing conditions with HA-agarose beads. The immunopellet was dissolved in Laemmli sample buffer containing β-mercaptoethanol and analyzed by Western blotting (WB) using an anti-V5 antibody. Western blot analysis of the inputs is shown below. Asterisk, signal corresponds to primary antibody used in the immunoprecipitation (IP).
Figure 5. Forced dimerization of PrP^C interferes with propagation of PrP^Sc.

A and C, persistently infected 22L-ScN2a cells were transiently transfected with the constructs indicated. Cell lysates were prepared and subjected to PK digestion (+PK) or left untreated (−PK) prior to immunoblot analysis using the monoclonal anti-PrP antibody 3F4 to exclusively detect the transfected PrP but not the endogenous PrP^C (A) or using the monoclonal anti-PrP antibody 4H11 to detect endogenous mouse PrP^C and PrP^Sc in addition (C). B and D, quantitative analysis of the amount of 3F4-positive (B) and total (D) PrP^Sc in transfected 22L-ScN2a cells. The relative (rel.) amount of PrP and PK-resistant PrP^Sc was measured densitometrically using ImageJ software. The relative amount of PK-resistant PrP^Sc present in cells expressing transfected WTPrP^C (C) or control-transfected cells (D) was set as 1. Data represent mean ± S.D. of 3 independent experiments.

E, putative model of the protective activity of PrP^C dimers. Under physiological conditions, PrP^C forms a dimer. Upon dissociation, PrP^C monomers interact with and are converted by PrP^Sc. PrP dimers may interact with PrP^Sc, but conversion does not occur. In case PrP^C dimers bind to PrP^Sc with a higher affinity than monomeric PrP^C, interaction of monomeric PrP^C with PrP^Sc is decreased, and its conversion is reduced. Alternatively or in addition, PrP^Sc in complex with PrP^C dimers is subjected to increased intracellular degradation. Error bars represent S.D.
tific), and murine N2a cells were cultured in minimum essential medium (MEM; Thermo Fisher Scientific), both with the addition of 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in a humidified 5% CO₂ atmosphere at 37 °C. Cells cultivated on a 3.5-cm cell culture dish (Nunc, Roskilde, Denmark) were transfected with plasmid DNA by a liposome-mediated method using Lipofectamine® LTX and PLUS™ reagent (Life Technologies) according to the manufacturer’s instructions. After 24 h, cells were washed twice with cold phosphate-buffered saline (PBS), scraped off the plate, pelleted by centrifugation (5,000 × g, 5 min), and lysed in detergent buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS). The cell lysates were either analyzed directly or centrifuged (20,000 × g, 10 min) to analyze the postnuclear supernatant.

N2a cells persistently infected with the mouse prions strain 22L (22L-ScN2a) (28) were cultivated in Opti-MEM GlutaMAX (Gibco). 22L-ScN2a cells were transfected using Lipofectamine® LTX and PLUS™ reagent according to the manufacturer’s protocol. Briefly, 1 × 10⁸ cells per plate were cultured in 10-cm plates. Plasmid (3 µg) and PLUS™ reagent were incubated along with Opti-MEM medium for 5 min and then added to the mixture of Lipofectamine® LTX reagent and Opti-MEM. The solution was kept for 15 min at room temperature. This solution was added drop by drop to cells and gently mixed. Cells were incubated at 37 °C. After 48 h, a second transfection was done as described above. 96 h after the first transfection, cells were lysed in cold lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate) for 10 min. One half of the lysate from each plate was incubated with PK at a final concentration of 20 µg/ml for 30 min at 37 °C. Proteinase inhibitor (0.5 mM Pefabloc) was added to inhibit PK digestion, and samples were precipitated in methanol. To the untreated sample, Pefabloc was added before methanol precipitation. Precipitated proteins were resuspended in TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA).

Deglycosylation (PNGaseF and EndoH), phospholipase C treatment, and trypsin digestion

To deglycosylate proteins, cell lysates were treated with PNGaseF or endoglycosidase H for 1 h at 37 °C according to the manufacturer’s instructions. For PIPLC treatment, cells were washed twice with PBS. PIPLC diluted in PBS was added to the cells for 4 h at 37 °C. Secreted PrP was precipitated by trichloroacetic acid (TCA) and analyzed by Western blotting. To analyze the localization of the prion protein and the generated mutants, cells were digested with trypsin, a member of the serine protease S1 family that digests cell surface proteins. Cells were washed twice with PBS and treated with trypsin for 25 min at room temperature. The reaction was terminated by the addition of Complete Mini EDTA-free Protease Inhibitor Mixture. Cell lysates were further analyzed by Western blotting.

Coimmunoprecipitation

To analyze formation of dimers, N2a or HeLa cells were cotransfected with the indicated constructs (V5- or HA-tagged) and lysed in detergent buffer (0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate in PBS). Postnuclear supernatants were incubated with anti-HA-agarose beads under nonreducing conditions in the case of disulfide-stabilized coimmunoprecipitation and under reducing conditions for the native coimmunoprecipitation (overnight, 4 °C, rotating). The immunocomplex was washed with lysis buffer and PBS and further analyzed by Western blotting.

Western blotting

For Western blot analysis, lysates were boiled in Laemmli sample buffer with or without β-mercaptoethanol (4%, v/v). Following SDS-PAGE, proteins were transferred to nitrocellulose by electrobolting. Membranes were blocked by incubation in TBS-T (TBS with 0.1% (w/v) Tween 20) containing 5% skimmed milk for 1 h at room temperature and incubated with primary antibody in TBS-T + 5% skimmed milk for 18 h at 4 °C. After washing with TBS-T, blots were incubated with respective secondary antibody (IRDye-IR Technology, LI-COR Biosciences; or HRP) in TBS-T for 1 h at room temperature. Protein signals were visualized using an Odyssey® 9120 scanner. Peroxidase activity was detected by enhanced chemiluminescence (ECL) (Promega).

To analyze PrP in persistently scrapie-infected 22L-ScN2a cells, Western blot analysis was performed as described previously (43). Briefly, samples (± PK) were subjected to 12.5% SDS-PAGE and electrobolotted on Hybond P 0.45-µm PVDF membranes (Amersham Biosciences). Anti-PrP mAb 3F4 or 4H11 was used as primary antibody, and goat anti-mouse HRP antibody was used as secondary antibody. The detection of signal in the immunoblot was done using Luminata Western chemiluminescent HRP substrate (Millipore).

Quantification

Dimerization efficiency of the constructs was measured densitometrically (Image Studio Lite) as the percentage of dimer fraction relative to total protein amount. In ScN2a cells, the amount of total PrP and PrPSc was also measured densitometrically (Image software) and the relative amount of PrPSc present in cells expressing WTPrPSc was set as 1. Data represent mean ± S.D. of ≥3 independent experiments. The standard deviation was determined using a Student’s t test (*, p < 0.05; ns, not significant).

Immunofluorescence analysis

Transiently transfected HeLa cells were grown on glass cover slips and fixed 24 h after transfection with 4% paraformaldehyde (10 min). One set was permeabilized (0.2% Triton in PBS), and one set was left unpermeabilized. Both sets were blocked in PBS with 5% normal goat serum for 1 h and incubated with anti-3F4 antibody overnight at 4 °C (in PBS containing 5% normal goat serum). After washing with PBS, incubation with the Cy3-conjugated anti-mouse secondary antibody (Alexa Fluor 488) followed for 1 h. Cells were mounted onto glass slides (with Fluoromount-G, Thermo Fisher Scientific) and examined by fluorescence microscopy (Zeiss ELYRA PS.1 and LSM 880).
PrP dimerization inhibits prion formation


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References


